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Understanding the complex macrophage landscape in MASLD

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Summary

Metabolic dysfunction-associated steatotic liver disease (MASLD) represents a spectrum of disease states ranging from simple steatosis to metabolic dysfunction-associated steatohepatitis (MASH), which can eventually lead to the development of cirrhosis and hepatocellular carcinoma. Macrophages have long been implicated in driving the progression from steatosis to end-stage disease, yet we still know relatively little about the precise involvement of these cells in MASLD progression and/or regression. Rather, there are a considerable number of conflicting reports regarding the precise roles of these cells. This confusion stems from the fact that, until recently, macrophages in the liver were considered a homogenous population. However, thanks to recent technological advances including multi-parameter flow cytometry, single-cell RNA sequencing and spatial proteogenomics, we now know that this is not the case. Rather hepatic macrophages, even in the healthy liver, are heterogenous, existing in multiple subsets with distinct transcriptional profiles and hence likely functions. This heterogeneity is even more prominent in MASLD, where the macrophage pool consists of multiple different subsets of resident and recruited cells. To probe the unique functions of these cells and determine if targeting macrophages may be a viable therapeutic strategy in MASLD, we first need to unravel this complexity and decipher which populations and/or activation states are present and what functions each of these may play in driving MASLD progression. In this review, we summarise recent advances in the field, highlighting what is currently known about the hepatic macrophage landscape in MASLD and the questions that remain to be tackled.

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Tissue-resident macrophages

Macrophages, first identified by Élie Metchnikoff in 1884 are a key component of the innate immune system.1 Best known for their role in immune surveillance, these cells are widespread throughout the body, where they are equipped with a plethora of pattern recognition receptors, allowing them to constantly probe their local environment and quickly sense and respond to both pathogen- and damage-associated molecular patterns. Upon sensing damaged or dying cells or other antigens, macrophages subsequently engulf these agents via phagocytosis, aided through their expression of various phagocytic receptors.[2–4] In addition to their core role in immune surveillance, tissue-resident macrophages also contribute to many other processes, including development and tissue homeostasis. For example, tissue-resident macrophages in the brain (microglia) also function to safeguard neuronal synapses,[5] the tissue-resident macrophages in the lung alveoli (alveolar macrophages) clear excess surfactant protein helping us to breathe,[6,7] while Kupffer cells (KCs), the resident macrophages of the liver have been proposed to function in lipid and iron homeostasis.[8,9] These distinctive functions highlight the exceptional adaptability and contributory capacity of tissue-resident macrophages within their host tissues, positioning macrophages as integral components across various organs. While originally proposed by Van Furth *et al.* to be part of the mononuclear phagocyte system and hence derived exclusively from circulating monocytes,[10] the ontogeny of macrophages has been the subject of intense investigation in recent years (reviewed extensively elsewhere[11,12]). Briefly, in mice, we now understand that starting from embryonic day 8.5 (E8.5), yolk sac erythro-myeloid progenitors generate pre-macrophages, which then migrate to embryonic tissues where they differentiate into tissue-specific macrophages during the process of embryogenesis.[11,12] In addition, erythro-myeloid progenitors migrate to the foetal liver, where they also produce so-called foetal liver monocytes that further contribute to the pool of tissue-resident macrophages during development.[11,12] In certain organs, such as the brain and the liver, under homeostatic conditions macrophages are long-lived and self-renewing, and hence persist into adulthood, with limited input from bone marrow-derived monocytes.[11,12] For other organs, like the heart, pancreas, or gut, fate-mapping approaches have shown that in adulthood, the pool of tissue-resident macrophages also originates from circulating monocytes, as these macrophages are continuously replaced throughout the organism's lifespan.[11,12] In non-homeostatic contexts however, macrophage ontogeny becomes more complex as bone marrow (BM)-derived monocytes massively infiltrate the injured or inflamed tissue, where they can then differentiate into many different macrophage subsets.[13,14]

Over the last decade, macrophages have been considered highly plastic cells, capable of rapidly responding to changes in the tissue by adopting different activation states or phenotypes

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Keypoints

- Not all macrophages in the liver are Kupffer cells.
- Kupffer cells can be distinguished from other hepatic macrophages based on their expression of a range of conserved markers including VSIG4, TIM4 and FOLR2.
- The hepatic macrophage landscape in MASLD is highly heterogeneous, consisting of different resident and recruited macrophage subsets.
- The precise roles played by the different macrophage subsets in MASLD remain under investigation.
- Lipid-associated macrophages represent a unique phenotype of recruited macrophages in MASLD but are also found across distinct disease contexts.
- The precise functions of lipid-associated macrophages remain under investigation, however most evidence to date suggests they may exert a protective effect in MASLD.

as needed[15,16] (Fig. 1). This perception mainly stems from the fact that *in vitro* BM-derived macrophages can be easily polarized into different activation states depending on the stimuli provided,[17] and macrophage populations *in vivo* exhibit different responses depending on the specific stimuli present in their local environment.[17–19] However, the recent insight into the origin and heterogeneity of macrophages within tissues has brought the concept of macrophage plasticity *in vivo* into question[16,20] and has raised the possibility that the perceived plasticity of macrophages is instead mirroring the presence of different macrophage populations, with different origins and different functions. The delineation of specific roles between resident and recruited macrophages during inflammation and their subsequent functions upon restoration of homeostasis has been the subject of extensive investigation over the last years.[13,21] However, our ability to discern the identity of recruited *vs*. resident macrophages and therefore assess their potential unique features has been hampered by the lack of markers enabling the different macrophage populations to be distinguished.[16] Thanks to the recent technological advances in multi-parameter flow cytometry/mass cytometry and single-cell RNA sequencing (scRNA-seq), we are now beginning to understand macrophage heterogeneity and how different macrophage populations with different ontogenies, activation states, and profiles function in different contexts. This is also true of our understanding of hepatic macrophage heterogeneity, where in recent years considerable research effort has been dedicated to understanding the functional heterogeneity of macrophages in the context of metabolic dysfunction-associated steatotic liver disease (MASLD).[22–29] Thus, in this review, we aim to summarise these findings, providing an overview of our current understanding of the hepatic macrophage landscape in MASLD.

MASLD

MASLD is the new nomenclature for the hepatic manifestation of metabolic syndrome which has previously been referred to as non-alcoholic fatty liver disease (NAFLD) or metabolic dysfunction-associated fatty liver disease (MAFLD).[30,31] MASLD is the most common chronic liver disease in the world, affecting 1.5 billion individuals worldwide.[32,33] With a prevalence of 25% to 30% in Western society, MASLD is associated with obesity, type 2 diabetes, and cardiovascular disease.[34] MASLD encompasses different stages of liver disease with different prognoses, ranging from simple and mainly asymptomatic steatosis (lipid accumulation in hepatocytes) to metabolic dysfunction-associated steatohepatitis (MASH, previously called NASH), characterised by increased inflammation and fibrosis, which can eventually lead to the development of cirrhosis and even hepatocellular carcinoma.[35,36] Despite significant research efforts, only one drug, resmetirom, has been approved for the treatment of MASLD[37] and while glucagon-like peptide-1 receptor agonists, including semaglutide, tirzepatide, and liraglutide have also shown encouraging results in recent clinical trials for the treatment of MASLD,[38–41] their approval for this indication is still pending. Consequently, liver transplantation remains the standard of care for most patients, and as such, given the current obesity epidemic and the lack of diagnostic tools, MASLD is predicted to become the leading indication for liver transplantation before 2030.[42–44] One key aspect of MASLD is that not all patients with simple steatosis will progress to MASH.[35] While the reasons for this dichotomy remain unclear, the activation of hepatic macrophages has been proposed to play a key role in driving this progression, as activated macrophages are proposed to activate hepatic stellate cells (HSCs), leading to their trans-differentiation into collagen-producing myofibroblasts, which drive the fibrosis characteristic of MASH.[45] However, as will be discussed throughout this review, the precise roles of macrophages in regulating MASLD progression remain unclear. The first evidence linking macrophage activation with the progression of MASLD comes from observations that macrophages accumulate in the liver as the disease develops,[45,46] but over the last decade these macrophages have been attributed with different, and often contradictory functions[47] (Fig. 1). Some studies suggest that macrophages are activated towards a pro-inflammatory phenotype, driven by various signals including excess lipids, liver damage, and signals from the intestine, thereby promoting MASLD progression.[48–50] Others however, attribute anti-inflammatory and pro-repair capacities to hepatic macrophages, suggesting a protective role in MASLD.[51–53] The main limitation of these early studies is that macrophages have been identified using generic pan-macrophage markers such as CD64 and F4/80, which hamper our ability to discern the potential heterogeneity present within the hepatic macrophage pool[8] (Fig. 1). Now armed with new tools and technologies, we have finally been able to dissect the different macrophage populations present in MASLD, enabling (ongoing) studies of their unique contributions to this disease.

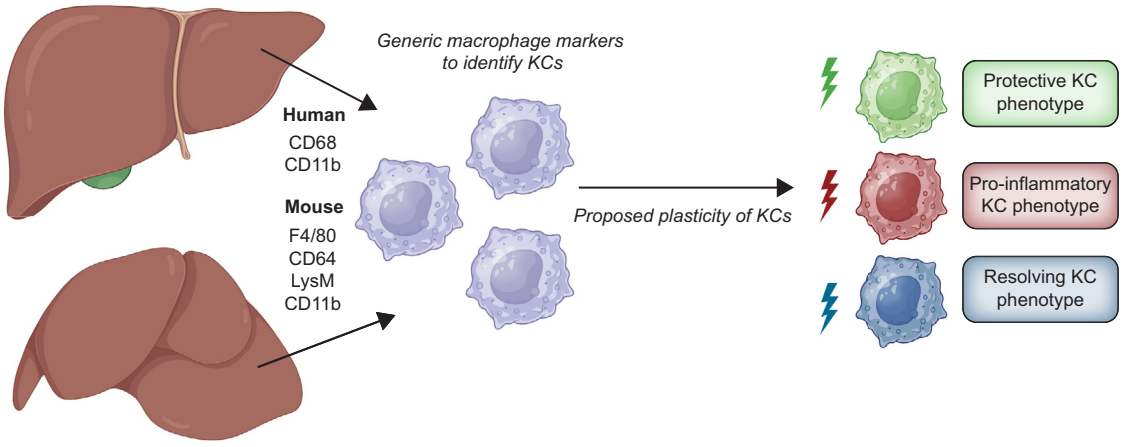

Fig. 1. A historical view of hepatic macrophages. Prior to the development of techniques allowing macrophage heterogeneity to be dissected, it was common practice to identify all macrophages in the liver as KCs using non-specific generic macrophage markers including CD68 and CD11b (human) and F4/80, CD64, LysM and CD11b (mouse). As such KCs were proposed to have many different phenotypes and hence contradictory functions in MASLD depending on the stimuli received. However, this perceived idea of plasticity in tissue-resident macrophages such as KCs is currently being challenged, with the plasticity now proposed to be related to the presence of distinct subsets of macrophages. KCs, Kupffer cells; MASLD, metabolic dysfunction-associated steatotic liver disease.

Kupffer cells; the resident macrophages of the liver

The healthy liver harbours one of the largest populations of tissue-resident macrophages in the body (Fig. 2). These cells, called Kupffer cells (KCs), which account for >90% of all liver macrophages under homeostatic conditions, were first identified in 1876 by Wilhelm von Kupffer.[54] Ontogenically, KCs are derived during embryogenesis from yolk-sac macrophages and foetal liver monocyte progenitors, being primarily of foetal liver

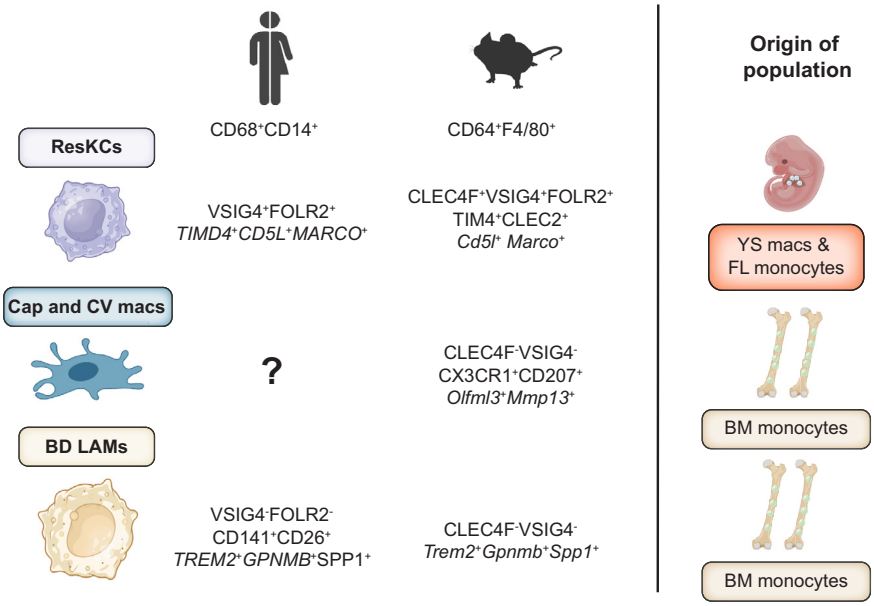

Fig. 2. An updated view of macrophage heterogeneity in the healthy liver. With the advent of new technologies including scRNA-seq and multi-parameter flow cytometry and confocal microscopy, we now have a much deeper understanding of hepatic macrophage heterogeneity under homeostatic conditions. Whereas previously all macrophages expressing generic macrophage markers were considered KCs, we now know that these cells can be subdivided into at least three subsets based on surface marker and gene expression profiles. The majority of macrophages in the healthy liver are indeed KCs derived during embryogenesis from yolk-sac macrophages (YS macs) and foetal liver (FL) monocytes, which can be identified with the indicated markers in mice and humans. In addition to the KCs, two smaller subsets of macrophages exist in distinct niches, the capsule and CV macrophages which reside in the liver capsule and close to the CV and the BD-LAMs, which, as their name suggests, reside in close proximity to bile ducts. Both populations have been shown to arise from BM monocytes. Unfortunately, our ability to identify these populations is more limited, often relying on gene expression rather than surface protein markers. Moreover, in humans, markers of capsule and CV macrophages remains to be determined. BD, bile duct; BM, bone marrow; CV, central vein; KCs, Kupffer cells; LAMs, lipid-associated macrophages; ResKCs, resident KCs.

monocyte origin.[11] At least in mice, these embryonic KCs are long-lived, persisting into adulthood due to their capacity for self-renewal, largely without any significant input from BM-derived monocytes.[11] An exception to this occurs during a brief temporal window in the early weeks of life when the growing liver allows for the recruitment and differentiation of BM-derived monocytes into KCs.[11,55]

KCs are found throughout the liver, although they are enriched in peri-portal regions.[28,56] They reside with the majority of their cell body within the liver sinusoids in close contact with liver sinusoidal endothelial cells (LSECs); however, they also extend multiple protrusions out of the sinusoids into the space of Disse, to contact and exchange materials with HSCs and hepatocytes.[57] As a result of this localisation, these (and likely other) local cellular interactions between KCs and other hepatic cells, collectively referred to as cells of the "KC niche", imprint and maintain KC identity.[8,57–60] By identity, we refer to the transcriptional profile of KCs, allowing them to be easily distinguished from other tissue-resident macrophage populations across the body and from other hepatic cells. Highlighting the relevance of the KC:KC-niche interactions, this identity has been shown to be highly conserved across species, enabling the definition of a core KC gene signature including *CD5L, VSIG4, MARCO, CD163, FOLR2, TIMD4, GFRA2, TMEM26, SLC40A1, HMOX1* and *VCAM1*[8,28] (Fig. 2). In mice and rats, but not humans, KCs can also be identified by their expression of the C-type lectin CLEC4F,[55,61,62] which is not expressed by any other cell population in the adult mouse and has been proposed to mediate the processing of desialylated platelets *in vivo*.[63] This unique expression of CLEC4F by murine KCs has enabled the generation of now widely used mouse models which allow for the specific targeting of KCs including *Clec4f* ^{Dtr}[55],*Clec4f* ^{Cre}[60,64] and *Clec4f* $^{Cre\text{-}ert2}$[65] mice. Note, however, that the targeting is not 100% specific to KCs, as the *Clec4f*^{Cre} animals also target a small population of B cells, for a yet unknown reason.[64] Across species, KC identity is regulated, at least in part, by their expression of the transcription factors liver X receptor-α (LXRα) and ID3.[57,64,66] LSECs induce and sustain the expression of LXRα through DLL4-dependent activation of the Notch signalling pathway, whereas hepatocytes are proposed to induce the expression of ID3.[57,60] Moreover, HSCs sustain KC identity through a conserved axis between ALK1 (activin receptor-like kinase) expressed by KCs, and BMP 9/10 (bone morphogenic proteins) expressed by HSCs.[28,67] Disruption of this ALK1-BMP9/10 axis leads to an almost complete loss of VSIG4^{+} KCs.[28,67] Additionally, HSCs are the main producers of IL-34 and colony-stimulating factor-1 (CSF1),[57] two macrophage growth factors that likely sustain KC survival and proliferation, although the unique contributions of each of these growth factors in regulating KC numbers remains to be accurately dissected.[59] In addition to these interactions, KC:immune cell interactions may also be relevant in imprinting KC identity or regulating their functions. For example, B cells closely interact with KCs in the homeostatic liver,[28] but the precise relevance of this interaction remains to be determined.

KC function is closely linked with their identity. As mentioned above, KCs are preferentially located in peri-portal (zone 1) regions of the liver,[28,56] meaning they are strategically positioned to recognise any harmful antigens draining from the intestine via the portal vein.[56] This preferential location is proposed to be induced by MYD88-dependent signalling imposed by commensal bacteria in LSECs, and as such is completely abrogated in germ-free animals.[56] Besides their role as professional phagocytes (Fig. 3), KCs possess key roles in iron metabolism, and express Fc receptors and scavenger receptors enabling them to eliminate damaged erythrocytes from the bloodstream[8,68,69] (Fig. 3). Additionally, KCs have a gene expression profile particularly enriched with genes associated with the uptake, processing, and export of surplus cholesterol.[8,9,70] This indicates a key role for KCs in transferring excess cholesterol to extracellular high-density lipoprotein acceptors, facilitating its transportation to hepatocytes (Fig. 3).

KCs in the homeostatic liver have typically been considered a homogeneous population. Fitting with this, there is no clear evidence of zonated gene expression profiles within KCs unlike observations in hepatocytes and LSECs.[28,71] However, recently, the homogeneity of KCs in the healthy liver has been questioned, with two studies proposing the existence of two populations of KCs, referred to as KC1s and KC2s. In these

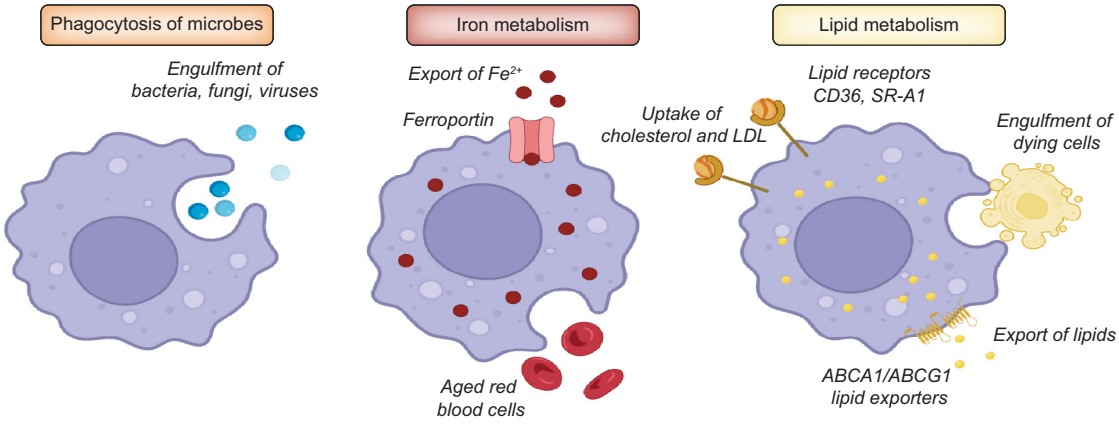

Fig. 3. KC functions in the healthy liver. Based on their unique transcriptional profile, KCs have been implicated in many functions. Perhaps the best studied of these is their role as professional phagocytes, where they actively phagocytose microbes including bacteria, viruses and fungi alongside dead and damaged cells and other inert antigens. Moreover, these cells are suggested to play roles in iron and lipid metabolism based on their expression of genes involved in uptake, metabolism and export of these molecules.

studies, KC2s represent ~20-25% of all KCs and can be identified amongst total KCs by their expression of ESAM and CD206, two markers typically associated with LSECs.[72,73] Functionally, KC2s have been implicated in metabolism and in regulating T-cell responses in viral hepatitis.[72,73] However, the existence of KC2s remains debated in the field.[28,74] The main reason for this contention is that while differentially expressed genes exist between ESAM$^-$CD206$^-$ KC1s and ESAM$^+$CD206$^+$ KC2s, all the specific genes associated with KC2s are also expressed by LSECs, suggesting that the KC2 subset may represent a doublet between KCs and LSECs (most probably resulting from incomplete tissue digestion), rather than a *bona fide* subpopulation of KCs.[28,73,74] Given the significant overlap between KCs and LSECs in space *in situ*,[57] this is not an easy puzzle to solve and thus further research, likely involving the use of novel spatial proteogenomic pipelines, is needed to clarify the true nature of these cells. Additionally, it has recently been reported that KCs could be subdivided in the healthy liver based on their expression of MARCO and IL10, as a subpopulation of MARCO$^+$IL10$^+$ KCs (independent of the KC1/KC2 nomenclature) has been reported to be present in the peri-portal space, which are crucial for limiting bacterial translocation and inflammation.[75] However, given that (at least at the mRNA level) human KCs have been reported to uniformly express *MARCO*[8,28], the precise relevance of these findings for human disease requires further investigation.[76]

Kupffer cells in MASLD

A characteristic hallmark of liver inflammation is the decline or depletion of the resident KC (ResKC) population.[8] This has been reported in MASLD and MASH,[22–24,27] but also in inflammatory settings including acute liver injury, bacterial infection, hepatocellular carcinoma, and cirrhosis (reviewed in[8]). The precise degree of this ResKC loss appears to be related to the severity of the injury/inflammation, with increased ResKC loss correlating with worse disease. Indeed, the depletion of tissue-resident macrophages, or the so-called macrophage-disappearance reaction is a characteristic of many organs in non-homeostatic contexts.[77,78] However, it is worth noting that in numerous studies the reduction of KCs has been quantified proportionally rather than as an absolute number. Moreover, as many of these studies have identified KCs based on their expression of generic macrophage markers including F4/80, CD64 and CD68, which are not exclusive to KCs (Figs 1,2), this suggests that the observed decline in KCs might, in fact, reflect a generic disappearance of macrophages, and the precise population undergoing reduction requires further evaluation. Such evaluation has been conducted in the context of MASLD, where a consensus decline of KCs is observed amongst total macrophages (CD64$^+$F4/80$^+$) when markers that within the total macrophage pool only identify KCs (including CLEC4F, VSIG4, CLEC2) or ResKCs specifically (TIM4) are used[22–24,27] (Fig. 4). However, the reasons underlying the loss of ResKCs remain incompletely understood.

One of the common hypotheses explaining this loss, especially in the context of MASLD, is that ResKCs would be activated by the altered environment and that this activation would drive the progression of MASLD.[22–24,27] However, considering the concept that ResKCs may not be as plastic as once thought (as discussed above), it is plausible to think that the ResKCs would be unable to sustain such an activated profile long-term, potentially leading to their demise. Indeed, numerous studies have reported that KCs can be activated by MASLD-related stimuli such as fatty acids, cholesterol, and lipid metabolites, as well as extracellular vesicles and damage-associated molecular patterns from steatotic hepatocytes.[47,79–81] This activation often results in the upregulation of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6, or chemokines like CCL2, CCL5, and CXCL12, which augment monocyte and neutrophil infiltration, fostering an inflammatory milieu in the MASLD liver.[47] These activated macrophages have been proposed to subsequently orchestrate MASLD progression by promoting liver steatosis via inhibition of the Peroxisome proliferator–activated receptor-α pathway in hepatocytes[48] or mediating liver fibrosis by promoting HSC activation through TNFα and IL-1β.[82,83] However, many of these findings were made using macrophages either isolated from the liver in the absence of *bona fide* KC markers or derived *in vitro* from BM or following the use of non-specific depletion strategies including clodronate liposomes which affect more than just macrophages.[84] As these systems are far removed from the *in vivo* context, whereby the lack of signals from niche cells likely alters the transcriptional profile of the macrophages[57] it is difficult to relate these findings to the situation *in vivo* (Fig. 5). Moreover, advances in our understanding of macrophage heterogeneity, ontogeny and KC identity, have led to this concept of KC activation being contested (Fig. 5). Firstly, studies with detailed analysis of liver macrophages using more appropriate markers for KCs have revealed that these cells are not activated, at least in the prototypical sense (pro-inflammatory cytokines, M1/M2 markers *etc*.), in Western diet (WD)-induced MASLD mouse models.[22] Similarly, analysis of total liver macrophages in obese patients compared with lean controls did not find any obvious activation profile in these cells.[85] Secondly, multiple additional macrophage populations have now been identified in MASLD ([22,27] and discussed in detail below), which may explain the discrepancies in terms of macrophage activation if it is in fact these cells instead of *bona fide* KCs that display an activated phenotype. However, the concept of KC activation potentially leading to their death cannot be completely disregarded either. The use of many different dietary models to induce MASLD to various degrees[86] also complicates this issue (Fig. 5). Indeed, in some of the more severe models that induce MASH quickly and often in the absence of obesity, including the methionine- and choline-deficient (MCD) diet, KC activation has been reported, whereby *bona fide* KCs identified using CLEC2 and TIM4 expression were shown to express elevated levels of genes associated with a lipopolysaccharide (LPS) signature in MASH compared with controls.[24] Additionally, feeding mice the modified Amylin diet (AMLN) has been shown to alter the transcriptional profile of KCs, although these changes were not associated with prototypical immune activation.[23,25] Whether disease stage would also affect the activation of KCs in the human liver also remains to be investigated (Fig. 5). Thus, it is clear further studies are warranted to accurately assess the role of KC activation both in terms of KC loss and MASLD progression.

Aside from KC activation, there are other hypotheses for the loss of KCs in MASLD. Taking into consideration the KC niche

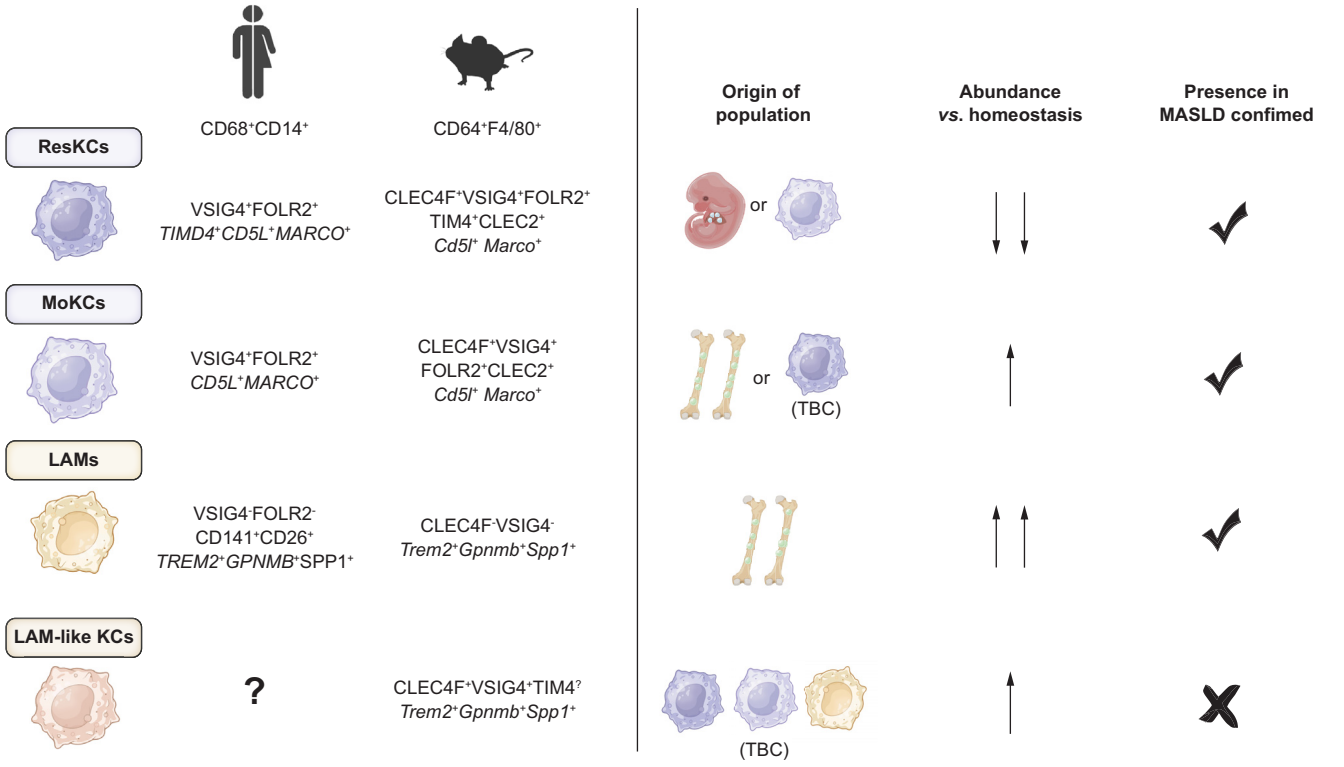

Fig. 4. An updated view of macrophage heterogeneity in MASLD. In the context of MASLD, the hepatic macrophage pool becomes even more heterogeneous. Firstly, a reduction in the number of ResKCs present compared with the healthy liver has been observed across models, although the severity of this reduction appears to be linked with disease stage (worse stage, less ResKCs). Moreover, bone marrow-derived monocytes get recruited which can then differentiate into either moKCs or LAMs. The fate of LAMs remains unclear, whereas at least in some models it has been reported that moKCs can (with time) acquire TIM4 expression and become indistinguishable from ResKCs. In a model of fibrosis, it has recently also been proposed that ResKCs may de-differentiate losing TIM4 to give rise to moKC-like cells, however, to date this has not been observed across multiple MASLD models. Finally, some studies suggest the presence of a hybrid LAM/KC population in the non-homeostatic liver, which herein we term LAM-like KCs. However, the ontogeny of these cells is not clear and moreover, their presence in MASLD remains to be validated. KCs, Kupffer cells; LAMs, lipid-associated macrophages; MASLD, metabolic dysfunction-associated steatotic liver disease; moKCs, monocyte-derived KCs; ResKCs, resident KCs; TBC, to be confirmed.

hypothesis,[58,59] one could postulate that the remodelling of the liver lobule upon injury—caused by collagen deposition, hepatocyte death, and immune cell infiltration—may create a short circuit within the niche, resulting in the loss of essential signals and cellular contacts crucial for the identity and survival of KCs, leading to their death. Indeed, increased KC death was reported in both the MCD and AMLN models of MASLD,[23,24] although we could not confirm this in the slower WD model,[22] possibly because of the slower loss of KCs. In keeping with this idea, LSECs change dramatically in MASLD, including the loss of their fenestrae and basement membrane,[87] which could influence their communication with KCs. Similarly, BMP9 production, a crucial growth factor involved in the maintenance of KCs, is also abrogated in MASLD.[88] As this factor is produced by HSCs,[28] this abrogation is probably a result of their trans-differentiation into myofibroblasts as is characteristic of liver fibrosis.[87,89] Additionally, scRNA-seq analysis of non-parenchymal cells from mice fed a WD for up to 36 weeks revealed several changes in their transcriptome that may affect communication with KCs, including increased expression of *Col1a2* by HSCs and *Cd36* and *Fabp4* by LSECs.[22] However, how these changes affect MASLD progression has not been directly investigated. Importantly, this crosstalk is likely bidirectional. Moreover, KCs altered by the presence of MASLD, may influence other hepatic cells, which in turn could further affect KC biology. For example, it has recently been reported that KCs inhibit HSC and hepatocyte activation through the production of miR-690-containing exosomes under homeostatic conditions, but miR-690 is lost during MASLD.[90] From this perspective, the hepatic niche represents a perfectly wired circuit in homeostasis that may falter during chronic injury, leading to the subsequent decline of ResKCs. This idea aligns with a recent model proposing the loss of tissue-resident macrophages during inflammation to be an "altruistic death", aimed at recruiting BM-derived macrophages better equipped to deal with the damage.[91] Collectively, this could illustrate a scenario where the hepatic niche, unable to sustain KC survival upon injury, facilitates the recruitment of new macrophages (Fig. 5). These new macrophages are discussed in more detail below.

Rather than their death, which is difficult to formally demonstrate because of issues in isolating and characterising dying cells, the decline in ResKCs could be due to their impaired proliferation or their trans-differentiation. Indeed, a lack of KC proliferation has been reported in MASLD and following *L. Monocytogenesis* infection.[92] However, it is important to mention that, at least in the WD-induced model of MASLD, this does not represent a loss of their ability to

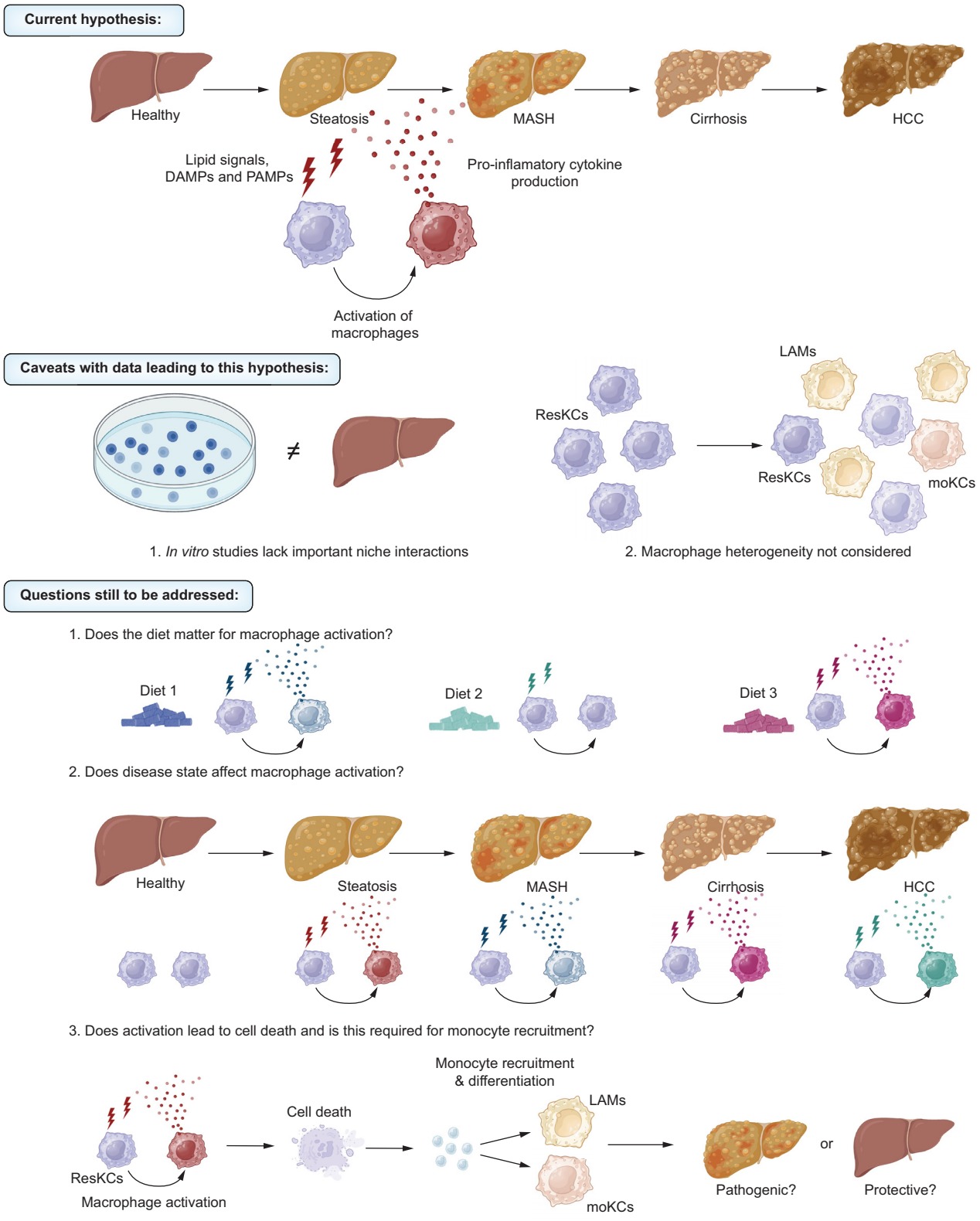

Fig. 5. KC activation in MASLD, an ongoing investigation. The widely accepted hypothesis is that the presence of hepatic steatosis would result in the activation of hepatic macrophages leading to their expression of pro-inflammatory cytokines which in turn would contribute to the progression of MASLD. While there is data available to support this claim, these often originate from *in vitro* studies which cannot mimic the complex cellular interplay observed in the MASLD liver or they are from *in vivo* studies where our current understanding of hepatic macrophage heterogeneity in MASLD (ResKCs, moKCs, LAMs) was not considered. Therefore, the validity of this hypothesis remains unclear. To be able to understand the role of macrophage activation in MASLD progression we will first need to answer a number of questions including (1) how dependent is this activation on the model of MASLD used? (2) Whether the activation of hepatic macrophages is related to the specific stage of MASLD? (3) How important is macrophage activation in regulating macrophage death and recruitment of monocytes which will then differentiate into other

proliferate as stimulation with a CSF1-Fc conjugate is sufficient to trigger KC proliferation.[22] As the levels of the typical macrophage growth factors IL-34 and CSF1, produced by HSCs and LSECs, respectively, were not found to be diminished in MASLD induced by WD feeding for 9 months,[22] the reasons for this lack of proliferation remain to be identified. However, alongside the caveats that mRNA expression does not always correlate with protein expression and that isolating structural cells from fibrotic livers can be challenging, it should be noted that the levels of these growth factors were measured by qPCR in isolated cells, hence a potential reduction in the availability of these growth factors cannot be completely ruled out[22]. More recently in the context of chronic fibrosis induced by administration of the chemical carbon tetrachloride, it was proposed that KCs may also transdifferentiate and lose expression of their identifying markers, including VSIG4 and TIM4.[93] This loss of key KC markers would result in their misclassification within the total macrophage pool as non-KCs and hence be observed as a loss of KCs (Fig. 4). However, to date, all fate-mapping studies performed in different diet-induced models of MASLD using different techniques including BM chimeras and transgenic reporter mice, suggest that the non-KCs identified are all of BM origin,[22–24,27] thus, arguing against such a de-differentiation of KCs in MASLD.

As alluded to above, MASLD is also characterised by the substantial recruitment of monocytes to the liver, which then undergo differentiation into BM-derived macrophages of different phenotypes (Fig. 4). Notably, similar populations of monocyte-derived macrophages have been reported across different murine models and in human MASLD,[28] highlighting their potential relevance. One fate that monocytes can take is to differentiate into monocyte-derived KCs (moKCs), and depending on the model used to induce MASLD, moKCs can account for 20-40% of total KCs. These cells were first reported following depletion of ResKCs using the *Clec4f* ^{Dtr} mouse model and following irradiation-induced loss of ResKCs.[55,94] Using the *Clec4f* ^{Dtr} model, the mechanisms of moKC recruitment have also been elucidated. Here, ResKC death leads to the release of TNFα and IL-1β, which activate HSCs and LSECs. This induces the expression of several genes involved in monocyte recruitment including *Ccl2*, *Ccl7, Cxcl10*, and *Pf4* and adhesion molecules that facilitate the arrest and diapedesis of monocytes including *Vcam1, Sele*, and *Icam1*.[57] Once recruited to the liver, KC identity is induced by LSECs, HSCs, and hepatocytes through the different signals described above.[57] Once generated, moKCs appear very similar to ResKCs in terms of their phenotype and transcriptome, expressing *bona fide* KC markers including CLEC4F, VSIG4 and FOLR2.[22,28,55] Acquisition of these markers takes approximately 3 days following monocyte recruitment to the liver.[22,55] Initially, moKCs lack expression of TIM4 and this feature is typically used to discriminate moKCs from ResKCs.[55] However, with time spent in the liver, moKCs can also acquire TIM4 expression (Fig. 4); in the *Clec4f* ^{Dtr} model, approximately 25% of moKCs were TIM4^{+} after 30 days.[55] This, along with the recent study in fibrosis which suggests ResKCs can lose TIM4 expression,[93] means care should be exercised when defining cells as moKCs or ResKCs based solely on expression of this marker. Rather, fate-mapping studies should be employed to more accurately discern the ontogeny of any KCs present in the liver in non-homeostatic contexts. Notably, while monocytes take approximately 3 days to acquire most KC markers, allowing them to be identified as moKCs, recently, CLEC2 has been identified as an additional early marker of macrophages that have entered the trajectory of KC differentiation.[22,24] The macrophages expressing CLEC2, but lacking expression of other KC markers have, as such, been termed pre-moKCs.[22] In the context of MASLD and MASH, it is unclear if similar mechanisms as those discussed above lead to moKC generation. However, as various studies have identified the mutual presence of ResKCs and moKCs,[22–24,27] this has enabled the comparison of their transcriptional profiles, which has shown that the degree of similarity between these two populations varies based on the model of MASLD used. In the WD and AMLN models, as observed following ResKC depletion under homeostatic conditions, ResKCs and moKCs in MASLD share a very similar profile,[22,23] although in the AMLN model this similar profile is distinct from that observed in ResKCs under homeostatic conditions, as the transcription factor ATF3 reprogrammes LXRα activity, downregulating many genes associated with KC identity.[23] However, in the harsher model of MCD diet-induced MASLD, moKCs (defined as CLEC2^{+}TIM4^{-} and thus also encompassing pre-moKCs) were reported to exhibit an overall pro-inflammatory profile and displayed a reduced efficiency in their ability to store triglycerides compared with ResKCs.[24] Interestingly, while moKCs are only observed in pathological settings or following experimental KC depletion in mice, in humans, moKCs have also been identified in the healthy liver in different scRNA-seq and single nuclei RNA-seq datasets.[28] This occurrence is likely attributed to the fact that we, as humans, do not live in specific pathogen-free conditions, thus over our lifetime we are often exposed to different microbes (both pathogenic and non-pathogenic) as well as various other factors including alcohol, fatty foods, toxins, and pharmaceutical agents that may trigger the need to replace ResKCs with moKCs. Despite the broad characterisation of the origin and transcriptomic profile of ResKCs and moKCs in MASLD, a precise function for these cells in the progression of the disease remains unclear. Moreover, as MASLD encompasses a spectrum of liver diseases associated with different degrees of inflammation, fibrosis, and damage, it is tempting to speculate that Res/moKCs might exert different functions based on the stage of the disease; however, this remains to be investigated.

macrophage subsets? These questions are critical because they will enable us to put the role of macrophage activation in context. If it is diet-specific, then perhaps certain models are better than others to study MASLD (depending on the activation status of these cells in human MASLD). Moreover, if macrophage activation is altered per disease stage or only occurs at specific disease stages, this can also shed light on the relevance of this phenomenon for MASLD progression. Finally, if activation leads to cell death, can this be utilised therapeutically? For example, can we prevent or promote cell death and/or only allow the right subsets of macrophages to be subsequently recruited? KCs, Kupffer cells; LAMs, lipid-associated macrophages; MASLD, metabolic dysfunction-associated steatotic liver disease; moKCs, monocyte-derived KCs; ResKCs, resident KCs.

Other macrophage populations in the healthy and MASLD liver

Alongside the ResKCs, the healthy liver is also home to a few smaller populations of macrophages, together contributing to approximately 10% of the total hepatic macrophage pool (Fig. 2). Liver capsule macrophages (LCMs) specifically reside in the liver capsule, a single layer of mesothelial cells and capsular fibroblasts located just beneath the surface of the liver.[28,95] In mice, LCMs express pan-macrophage markers such as F4/80 and CD64, however they lack the expression of KC-defining markers including VSIG4, TIM4 and CLEC4F. Instead, they express CX3CR1 and CD207.[28,95] However, it is worth noting that neither of these markers are unique to LCMs, as CD207 is also expressed by KCs, while CX3CR1 is also expressed by other non-KCs macrophages.[28] At the level of gene expression, murine LCMs can also be identified amongst other macrophages by their expression of *Olfml3* and *Mmp13*[28] (Fig. 2). In humans, while these cells have been observed using generic macrophage markers by confocal microscopy, we do not yet have a clear picture of their transcriptional profiles, likely because of the relative paucity of capsule material in liver biopsies.[28] Contrary to KCs, LCMs are constantly replenished from blood monocytes in adulthood.[95] Functionally, LCMs have been proposed to sense bacteria reaching the liver capsule and, in response, to recruit neutrophils.[95] Fitting with this, the depletion of LCMs increased liver pathogen load and abrogated neutrophil recruitment.[95] Here, LCM depletion was achieved with an anti-CSF1 receptor antibody, although this had no effect on KC numbers, effects on KC functions or on the other hepatic macrophage populations contributing to this phenotype cannot be ruled out.[95] Interestingly, a population of murine macrophages that share genes with LCMs have also been identified at the hepatic central vein; however, the specific functions of these macrophages require further investigation.[28] In addition to LCMs and central vein macrophages, an additional minor population of hepatic macrophages has been identified close to bile ducts in both mice and humans.[28] These BM-derived macrophages account for approximately 2-5% of total liver macrophages, express generic macrophage markers such as CD64 and F4/80, lack the expression of KC-defining markers and express a plethora of genes associated with lipid-associated macrophages (LAMs) first described in obese adipose tissue,[96] including *Spp1, Gpnmb, Trem2, Cd9*, *Fabp5, Cd63, Clec4d* and *Clec4e*.[28] In humans, these so-called bile duct LAMs (BD-LAMs) are also characterised by the expression of CD141 and CD26, which according to CITE-seq analysis can be used as surface markers for these cells[28] (Fig. 2). Functionally, BD-LAMs express more IL-1β at steady state than ResKCs, however, upon *in vivo* stimulation with LPS, BD-LAMs were less responsive than KCs, both in terms of pro- and anti-inflammatory cytokine production, suggesting that LAMs might be biased toward tolerance or perhaps desensitised to LPS given their proximity to the portal tract and the presence of gut-derived bacterial products (including LPS) that enter the liver at this location via the portal vein.[28]

As discussed above, in MASLD, the hepatic macrophage landscape becomes even more complex (Fig. 4). Alongside becoming moKCs, monocytes entering the MASLD liver can also become LAMs. Similar to homeostatic LAMs, these macrophages express generic macrophage markers, lack KC-defining markers and closely resemble LAMs from obese adipose tissue,[96] expressing *Spp1, Trem2, Gpnmb, Cd9, Fabp5* and *Clec4d*.[22,25,27,28,97,98] While relatively scarce in the healthy liver, upon the onset of inflammation, LAMs massively infiltrate the tissue, comprising up to 50% of total liver macrophages, again depending on the precise model used and the degree of inflammation (Box 1). Importantly, while these cells have been described across different models of MASLD in mice and in obese human livers, they are not always referred to as LAMs, causing some confusion. Indeed, these cells have also been referred to as scar-associated macrophages or SAMs,[97,98] NASH-associated macrophages or NAMs,[25] or instead simply labelled based on the genes they express, *e.g.* *Trem2*$^+$ macrophages[99–101] or *Spp1*$^+$ macrophages.[102] Irrespective of their precise name, all these macrophages exhibit remarkable similarities in terms of their gene expression profiles. Unfortunately, this naming problem is linked with the advances of scRNA-seq and the push to report novel cell types in publications and, as such, is not an issue unique to LAMs. Moving forward it is clear that a consensus on the most appropriate nomenclature for these cells is required. For the sake of convenience, in this review we will continue to refer to these cells as LAMs.

How does a monocyte entering the liver decide to become a moKC or a LAM? Spatial proteogenomic technologies have suggested that this fate decision is likely to be dictated by the location to which the monocytes are recruited and the local signals they encounter at that site.[28] Indeed, the level of injury across the liver lobule is often zonated, including regions with more pronounced damage compared with areas that remain relatively uncompromised.[22,28] In the context of MASLD, LAMs expand numerically and are preferentially localised in regions of steatosis and fibrosis, whereas moKCs are mostly found within the tissue that more closely resembles the healthy liver, within the sinusoids, aligning with a niche analogous to that occupied by ResKCs.[22,27,28] It is worth noting that the massive recruitment of monocytes to the injured tissue, where LAMs develop, suggests that LAMs are freshly recruited from the BM during inflammation rather than expanding from the BD-LAM population already present in the healthy liver. However, further investigation is needed to clarify this. Fitting with the location of LAMs in areas of steatosis and fibrosis, the efferocytosis of dying cells has been proposed to play a crucial role in imprinting the LAM phenotype.[100,103] This aligns with the observation that lipid stimulation *in vitro* drives the acquisition of the LAM phenotype on BM-derived macrophages.[28] In a mouse model of MASH, it has also been reported that “find me” signals produced by dying hepatocytes, such as hepatocyte-derived sphingosine-1-phosphate, are sufficient to drive the LAM phenotype *in vitro* through the interaction with sphingosine-1-phosphate receptor 1.[100] Collectively, these findings suggest that in MASLD, a combination of lipid accumulation, “find me” signals, and efferocytosis imprint the LAM phenotype on infiltrating macrophages. This implies that steatosis and fibrosis might constitute a distinct hepatic niche that steers the cellular fate of monocytes away from the KC identity. Importantly, the precise roles played by the different cells in the local environment in the recruitment of LAMs requires further investigation. Similarly, the precise transcription factors induced in recruited monocytes regulating LAM development also remains to be studied.

Box 1. Targeting LAMs as potential therapy.

While first identified in adipose tissue,[96] many reports have now identified the presence of LAMs across different organs and diseases. In the liver, LAMs have not only been identified in MASLD, but also in the context of acute liver injury induced by an overdose of paracetamol,[108] in cholangiopathies[109] and in human HCC.[110] Outside the liver, macrophages with a similar phenotype to LAMs have been identified in the inflamed heart,[111] atherosclerotic plaques,[112–114] Alzheimer's brain,[115–117] arthritic joints,[118,119] COVID-19-infected lung,[120] cancer,[103,121,122] dermis,[123] skeletal muscle,[124] and even in acne lesions.[125] This suggests that this macrophage phenotype is likely a global feature of tissue inflammation and/or injury across tissues and species. Interestingly, however, LAMs have been proposed to possess different roles based on the tissue and disease context. For instance, LAM-like microglia in the context of Alzheimer's disease are thought to have protective functions preventing β amyloid plaque deposition.[115] Similarly, LAMs are thought to reduce plaque progression and necrotic core formation in a mouse model of atherosclerosis.[114] On the contrary, LAMs are proposed to be detrimental in acne lesions, where they are activated toward a pro-inflammatory state,[125] and in non-small cell lung cancer, where LAM-like cells promote tumour growth by inhibiting NK cell activation.[103] Collectively, these observations indicate that the role of LAMs is likely contingent on the specific context and disease under investigation. This highlights the need for a thorough comparative analysis of LAMs across disease contexts to determine the precise nature of these cells in different organs, specifically how similar or distinct are they in terms of transcriptional signatures, phenotypes and functions. It is plausible that the widespread presence of LAMs in different diseases may represent a unique therapeutic opportunity. If a conserved program is operative in infiltrating macrophages during inflammation and/or injury, leading to the acquisition of the LAM phenotype, then deciphering the precise functions of LAMs could enable us to target these cells as a global therapeutic approach for many important human diseases.

HCC, hepatocellular carcinoma; LAMs, lipid-associated macrophages; MASLD, metabolic dysfunction-associated steatotic liver disease; NK, natural killer.

Despite the discovery of various factors imprinting the LAM identity, numerous questions remain regarding the biology of these cells. Specifically, these are related to the functions of LAMs and their fates upon resolution of inflammation. For example, do LAMs differentiate further into KCs or other macrophages or do they die? To date, there have been few studies investigating how the macrophage populations are altered by weight loss induced either by specific treatments or a return to normal chow. However, two studies using the MCD diet found that the non-KC macrophages (including LAMs) were almost completely lost from the liver in recovery, while moKCs were significantly reduced in number and ResKCs increased.[24,29] Notably, BM chimeras suggested that some BM-derived macrophages gave rise to ResKCs, although due to the lack of fate-mapping tools, we cannot yet fully address the origins (*e.g.* LAMs or moKCs) of these cells. Similarly, dietary and lifestyle interventions in patients with MASLD lowered the serum concentration of sCD163,[104] a macrophage marker, potentially reflecting a reduction in macrophages within the liver, although which macrophage population(s) were affected and if any of these reside in the liver as opposed to other organs such as adipose tissue remains unclear. While the fates of LAMs and indeed other hepatic macrophages require further study, the functions of LAMs have begun to be unravelled (Fig. 6). One gene expressed by LAMs, both in mice and humans, is the lipid receptor called triggering receptor expressed on myeloid cells-2 (*Trem2*).[22,28] Mice deficient in *Trem2* demonstrate exacerbated MASLD when fed a HFD[99,101]; this phenotype was associated with altered macrophage-hepatocyte cross-talk, whereby *Trem2*-deficient macrophages produced increased amounts of exosomes which interfered with hepatocyte mitochondrial function.[99] This was proposed to be dependent on the high presence of miR-106b-5p within those exosomes, which directly target mitofusin 2, a protein involved in mitochondrial fusion and metabolic adaptation.[99] *Trem2* has also been proposed to mediate macrophage efferocytosis of lipid-laden hepatocytes in MASLD, thereby suppressing inflammation and maintaining homeostasis in the steatotic liver.[100] However, under prolonged hyper-nutrition, the increased production of pro-inflammatory cytokines TNFα and IL-1β increased TREM2 shedding through ADAM17-dependent proteolytic cleavage.[100] This event has been proposed to drive an abnormal accumulation of dying hepatocytes that facilitate the progression to MASH.[100] In an additional report, *Trem2* deletion also increased the expression of profibrotic genes including *Tgfb1* in the liver, and *Trem2*-deficient BM-derived macrophages were found to possess pro-fibrogenic potential when incubated with fibroblasts *in vitro*.[101] Collectively, these studies suggest that *Trem2* expression is protective in MASLD (Fig. 6). However, none of these studies solely targeted *Trem2* in LAMs, relying instead on either whole-body knockout mice or *Lyz2*^{Cre} transgenic animals which target multiple myeloid populations (Fig. 6). Indeed, while some studies reported that *Trem2* expression in the liver is restricted to LAMs[22,100] others suggest that KCs can also upregulate *Trem2* upon injury, including in the AMLN diet-induced model of MASLD, perhaps by acquiring a LAM-like phenotype.[8,23,25] Thus, further study is required to distinguish the role of *Trem2* in LAMs from its role in other myeloid cells. Moreover, *Trem2* is only one of the many genes expressed by LAMs, thus it may not be solely responsible for LAM functionality. Indeed, LAMs identified in cirrhotic human liver biopsies are proposed to possess a pro-inflammatory phenotype based on *in vitro* and *in silico* analyses of their entire transcriptomic profile.[97] Moreover, LAMs also express osteopontin (encoded by *Spp1*),[22] which has been proposed to drive a fibrogenic response in the liver.[105] Indeed, a recent report identified a population of pro-fibrogenic *Spp1*-expressing macrophages that drive fibroblast activation in the kidney in a CXCL4-dependent manner, suggesting a pro-fibrotic role for LAMs.[106] Similarly, a recent report linked LAMs to a pro-fibrogenic function in both liver and lung fibrosis.[98] However, it is worth noting that to reach this conclusion, the authors combined blockade of TGFβ, IL-17 and GM-CSF which resulted in the concomitant reduction of LAMs and fibrosis.[98] As inhibiting these crucial cytokines is likely to impact more than just the LAMs, it is yet to be determined whether the decrease in fibrosis is a consequence of the absence of LAMs or if the diminished presence of LAMs is a direct outcome of restricted fibrosis or injury. In contrast, another study observed that while *Spp1*-expressing macrophages accumulate in human MASLD, these cells exhibit metabolic properties rather than inflammatory ones.[102] Consistent with this, conditional knock-in of *Spp1* in both myeloid cells (*Lyz2* ^{Cre}) and KCs (*Clec4f* ^{Cre}) conferred protection, whereas *Spp1* deletion from myeloid cells (*Lyz2* ^{Cre})

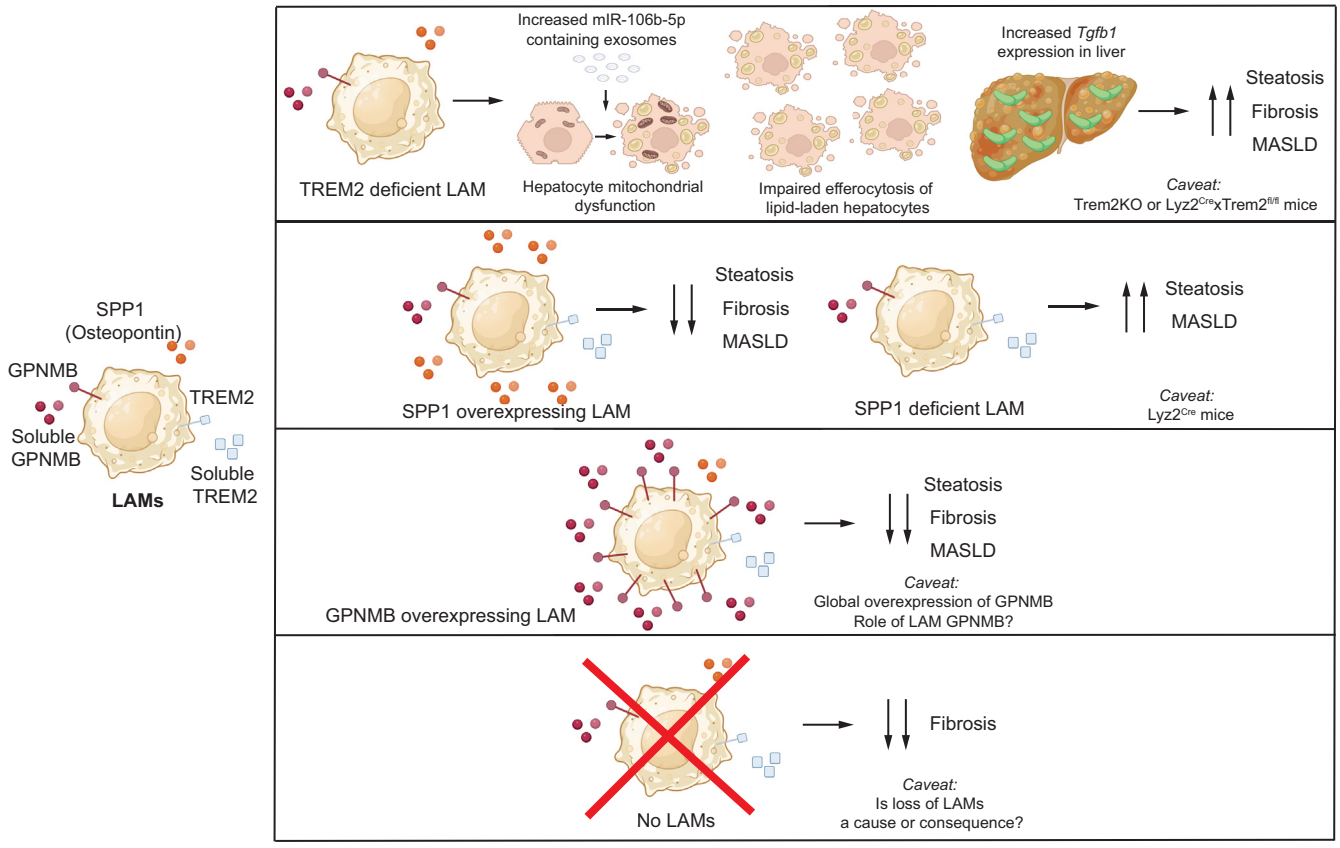

Fig. 6. LAM functions in MASLD. To date the study of LAM function remains in its infancy due to the lack of tools with which we can specifically study these cells. There is currently conflicting information surrounding their functions in the liver and across other tissues. Global loss of TREM2 or loss of TREM2 from myeloid cells (*Lyz2*^{Cre}) suggests that these cells may have a protective function in MASLD as a result of mediating hepatocyte-macrophage crosstalk, efferocytosis of lipid-laden hepatocytes and potentially also macrophage-fibroblast crosstalk. Similarly, studies manipulating expression levels of *Spp1* in myeloid cells (*Lyz2*^{Cre}) in MASLD have also suggested a protective role for LAMs, while global overexpression of *Gpnmb*, another core LAM gene, also resulted in ameliorated steatosis and fibrosis. Conversely, loss of LAMs achieved by global blockade of TGFβ, IL17 and GM-CSF led to improved fibrosis, suggesting a detrimental role for these cells. While it remains unclear in this setting if the loss of LAMs is a cause or consequence of improved fibrosis, these studies highlight the need for additional functional studies regarding the specific roles of LAMs in MASLD and fibrosis. LAMs, lipid-associated macrophages; MASLD, metabolic dysfunction-associated steatotic liver disease.

worsened MASLD progression in mice fed a WD[102] (Fig. 6). Moreover, another gene highly expressed by LAMs, *Gpnmb*, has been reported to be beneficial in MASLD, as global overexpression of *Gpnmb* ameliorates fat accumulation and fibrosis[107] (Fig. 6). Together, these reports highlight that a consensus on the function of LAMs remains to be reached, as different studies employing various tools and genes to investigate LAM functions have produced conflicting results (Fig. 6). Moreover, the possibility that KCs can acquire the LAM phenotype still requires additional investigation. If so, it will also be important to assess if LAMs and LAM-like KCs possess similar or divergent functions.

Conclusion

Taken together, it is clear that the hepatic macrophage landscape in MASLD is highly heterogeneous consisting of a combination of resident and recruited macrophages of different subsets. Not only that but within each subset, recent work suggests that distinct activation states may also exist, possibly depending on disease stage, like the putative LAM-like KCs. Understanding each of these subsets and/or activation states and their unique and conserved contributions to MASLD will be crucial if we are to be in a position to efficiently target macrophages for MASLD therapies.

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Abbreviations

AMLN, amylin; BM, bone marrow; Cd5l, CD5 molecule like; CLEC4F, C-type lectin domain family 4 member F; CSF1, colony-stimulating factor-1; Folr2, Folate receptor beta; HSCs, hepatic stellate cells; KCs, Kupffer cells; LAMs, lipid-associated macrophages; LCMs, liver capsule macrophages; LPS, lipopolysaccharide; LXRα, liver X receptor-α; Marco, Macrophage receptor with collagenous structure; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MCD, methionine- and

choline-deficient; moKCs, monocyte-derived KCs; NK, natural killer; ResKCs, resident KCs; scRNA-seq, single-cell RNA sequencing; SD, Standard diet; TIM4, T cell membrane protein 4; VSIG4, V-set and immunoglobulin domain containing 4; WD, Western diet.

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Conflict of interest

The authors of this study declare that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

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Supplementary data

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